Aldose Reductase and Its Inhibition in the Control of Diabetic Complications*

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ABSTRACT

Aldose reductase is a rate limiting enzyme in the polyol pathway associated with the conversion of glucose to sorbitol. The enzyme is located in the eye (cornea, retina, lens), kidney, myelin sheath, and also in other tissues less involved in diabetic complications. Experiments in diabetic animals have implicated sorbitol accumulation in the lens to the development of cataracts. The use of inhibitors of aldose reductase in animal studies has demonstrated that diabetic complications such as cataracts, nephropathy, and slowing of nerve conduction can be ameliorated. While an osmotic effect can explain the physical changes in the lens leading to cataract formation, the effect of sorbitol accumulation in other tissues and the resulting diabetic complications has been linked to the depletion of myoinositol content resulting in a derangement of sodium-potassium adenosine triphosphatase activity.

Since glucose and other hexoses are poor substrates for aldose reductase, it is only in hyperglycemia when the enzyme hexokinase is saturated that aldose reductase is activated, leading to accumulation of sorbitol. The kinetics of inhibition of aldose reductase by a variety of inhibitors has been delineated. The dose required varies from inhibitor to inhibitor and is consistent with their inhibition constants. Toxicity is a consideration in the use of some of the inhibitors, as was demonstrated with sorbinil which caused hypersensitivity reactions in 10 percent of patients. Other inhibitors such as tolrestat have shown efficacy and are under clinical investigation. Interpretation of results obtained with aldose reductase inhibitor therapy in human subjects suggest that these inhibitors are effective at early stages of diabetic complications.

Introduction

Aldose reductase or alditol: NADPH oxidoreductase (EC 1.1.1.21) is the rate-limiting enzyme of the polyol pathway (the term polyol refers to each carbon of the carbohydrate molecule bearing an hydroxyl group). The enzyme catalyzes the reduction of hexoses, such as glucose and galactose, to their corresponding alcohols, sorbitol and galactitol, respectively. Sorbitol in turn is oxidized by an
NAD\(^+\) dependent enzyme called sorbitol or polyol dehydrogenase (i-iditol dehydrogenase EC 1.1.1.14) to fructose. The polyol pathway as a source of fructose in the seminal vesicle was first demonstrated in 1956.\(^\text{16}\) Although sorbitol has been implicated in the osmoregulation of kidney tubules, the physiological significance of the polyol pathway in most tissues still remains to be established.\(^\text{6}\) Sorbitol dehydrogenase, the second enzyme in the polyol pathway, while having the ability to oxidize a variety of sugar alcohols, in addition to sorbitol, is inefficient in oxidizing galactitol, thus leading to its accumulation in galactosemia.\(^\text{12}\) The metabolism of glucose by the polyol pathway and the conversion of galactitol by aldose reductase is summarized in figure 1.

Significantly, and yet fortuitously, both glucose and galactose are poor substrates for aldose reductase, since the concentration of these substrates required to half-

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**GLUCOSE METABOLISM BY POLYOL PATHWAY**

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\begin{align*}
\text{CHO} & \quad \text{HCOH} \\
\text{HOCH} & \quad \text{HCOH} \\
\text{HCOH} & \quad \text{HCOH} \\
\text{HCOH} & \\
\text{H} & \\
\text{D-GLUCOSE} & \\
\end{align*}
\]

\[
\begin{align*}
\text{CHO} & \quad \text{HCOH} \\
\text{HOCH} & \quad \text{HCOH} \\
\text{HCOH} & \quad \text{HCOH} \\
\text{HCOH} & \quad \text{HCOH} \\
\text{H} & \\
\text{SORBITOL} & \\
\end{align*}
\]

\[
\begin{align*}
\text{CHO} & \quad \text{HCOH} \\
\text{HOCH} & \quad \text{HCOH} \\
\text{HCOH} & \quad \text{HCOH} \\
\text{HCOH} & \quad \text{HCOH} \\
\text{H} & \\
\text{D-FRUCTOSE} & \\
\end{align*}
\]

**GALACTOSE METABOLISM**

\[
\begin{align*}
\text{CHO} & \quad \text{HCOH} \\
\text{HOCH} & \quad \text{HCOH} \\
\text{HCOH} & \quad \text{HCOH} \\
\text{HCOH} & \quad \text{HCOH} \\
\text{H} & \\
\text{D - GALACTOSE} & \\
\end{align*}
\]

\[
\begin{align*}
\text{CHO} & \quad \text{HCOH} \\
\text{HOCH} & \quad \text{HCOH} \\
\text{HCOH} & \quad \text{HCOH} \\
\text{HCOH} & \quad \text{HCOH} \\
\text{H} & \\
\text{GALACTITOL} & \\
\end{align*}
\]

_Figure 1._ Glucose and galactose metabolism by polyol pathway. AR = aldose reductase. SDH = sorbitol dehydrogenase.
saturate the enzyme approximates between 70 to 150 mM (Km). Thus, under normoglycemic conditions, aldose reductase is not activated. However, in hyperglycemic conditions, such as in diabetes mellitus, when the enzyme hexokinase which converts glucose to glucose-6-phosphate is saturated, that aldose reductase is activated causing conversion of glucose to sorbitol. Since the activity of the enzyme sorbitol dehydrogenase is not proportionately increased, sorbitol accumulates within the cell. The intracellular accumulation of sorbitol is facilitated by the polar nature of this compound which limits its entry into the cell membrane. Pioneering studies carried out on animals have associated the accumulation of sorbitol to diabetic pathology in the eye (lens, cornea, retina), peripheral nerves, and kidney. The prevention or delaying of such complications has been suggested using inhibitors of aldose reductase.\textsuperscript{21,22,26}

**Tissue Distribution of Aldose Reductase and Diabetic Complications**

The presence of aldose reductase in the lens of the eye was first reported in 1959.\textsuperscript{30} Subsequent animal studies have clearly demonstrated in diabetic and galactose fed rats the progression of cataract formation through the accumulation of either sorbitol or galactitol.\textsuperscript{21} In the retina, aldose reductase has been localized in the retinal pericytes (mural cells) of intact retinal capillaries.\textsuperscript{2} Characteristic features of diabetic retinopathy include selective loss in the number of pericytes and thickening of the capillary basement membrane. The latter can be prevented by the administration of aldose reductase inhibitors.\textsuperscript{26}

The presence of aldose reductase in the corneal epithelium has also been demonstrated.\textsuperscript{21} Corneal changes (keratopathy) seen in diabetic and galactose fed rats range from a delay in corneal reepithelialization to a hazy edematous appearance of the cornea. These findings have been prevented by oral administration or topical application of aldose reductase inhibitors.\textsuperscript{21} The accumulation of either sorbitol or galactitol in the sciatic nerves of diabetic or galactose-fed rats demonstrated the presence of aldose reductase in the nerve and its role in neuropathy associated with a decrease in motor nerve conduction velocity.\textsuperscript{11} Thus, conditions associated with diabetic neuropathy, such as decreases in motor nerve conduction velocity and axonal transport, have been reversed in animal experiments by administration of aldose reductase inhibitors.\textsuperscript{20}

In the kidney, the highest concentrations of aldose reductase are found in the inner medulla and papilla, with lower concentrations in the cortex. The enzyme is precisely located in the tubular epithelial cells of the thin limbs of the loop of henle, the collecting tubules, the interstitial cells in the inner medulla, the glomerular epithelial cell or podocyte, the mesangial cells, and the epithelial cells of the distal convoluted tubules.\textsuperscript{22} Characteristically, diabetic nephropathy includes increased glomerular filtration rate, the thickening of the mesangial matrix, and an increase in the width of the glomerular basement membrane.\textsuperscript{25} Reduction of the increase in glomerular filtration rate, proteinuria, mesangial expansion, and glomerular basement membrane thickening have been effected in diabetic animals through use of aldose reductase inhibitors.\textsuperscript{22} The tissue localization of aldose reductase and related diabetic complications are summarized in table I.

**The Relationship Between Polyol Accumulation and Diabetic Complications: Osmotic Hypothesis**

Cataract formation has been related to an osmotic effect owing to the accumulation of polyols such as sorbitol in diabetic
According to this osmotic hypothesis or polyol theory, the increase in sorbitol and fructose concentration in the diabetic lens causes water to be drawn into the lens fibers, thus causing them to swell. This swelling alters cell membrane permeability and disrupts the normally high potassium to sodium ratio. As sodium enters the cell by diffusion, potassium is lost. Together with potassium, adenosine triphosphate (ATP), free aminoacids, myoinositol, and reduced glutathione levels decrease as they exit the cell. In contrast, as sodium and chloride levels within the cell increase, the lens swells. The swollen lens fibers then rupture to form vacuoles initiating cataract formation. In later stages, protein synthesis in the lens is affected, and the cataract progresses as vacuoles increase towards cortical opacification.

Finally, the lens membrane becomes freely permeable to all constituents except for the very large proteins, and the cataract progresses to the nuclear cataract stage. Although the osmotic hypothesis can explain events within the diabetic lens owing to polyol accumulation, it is not applicable to other issues where diabetic complications occur as a result of polyol accumulation. The latter is due to the fact that concentrations of sorbitol in tissues other than the lens (e.g., retina, glomerulus, and peripheral nerve) that are subject to diabetic complications are much less likely to cause water to be drawn into the cell and induce osmotically related changes. Instead alterations in the level of myoinositol is believed to result from accumulation of sorbitol and thus account for diabetic complications.

**MYOINOSITOL DEPLETION HYPOTHESIS**

There is evidence in diabetic neuropathy that elevated glucose levels compete for the carrier system needed to transport myoinositol into the nerve cell since glucose has a three dimensional configuration similar to myoinositol. Sorbitol accumulation is also believed to play a role in the depletion of myoinositol in the nerve cell, since the use of inhibitors for aldose reductase has been shown to prevent myoinositol depletion. Myoinositol needs to be incorporated into a class of membrane phospholipids called polyphosphoinositides. These phospholipids play a key role in the structure and control of functions of cell membrane. Of relevance to the myoinositol depletion hypothesis is the phosphorylated derivative of myoinositol called phosphatidylinositol 4,5 bisphosphate (PIP$_2$), the hydrolysis of which yields two key substances having second messenger properties: diacylglycerol and 1,4,5-inositol triphosphate.

One of the functions of diacylglycerol is to activate in the lipid cell membrane bilayer the enzyme protein kinase C which in turn activates sodium-potassium ATPase. Since the transport of myoinositol into the cell is dependent on sodium-potassium ATPase, limiting entry of myoinositol into the cell as a con-
sequence of hyperglycemia, increases aldose reductase activity and sorbitol accumulation, thus creating a cyclic chain of events that eventually depletes the cell of myoinositol. In figure 2 are summarized events leading to myoinositol depletion. As a consequence of myoinositol depletion in the peripheral nerve, there is an accumulation of sodium within the cytoplasm of the axon of the nerve cell causing a deficit in the generation of action potential or electrical nerve impulse.

Myoinositol depletion and reduction of sodium-potassium ATPase activity owing to sorbitol accumulation have been demonstrated in the glomeruli and the retina using diabetic animal models. However, some contradictory results have been reported, such as an increase in sodium potassium ATPase activity in the face of myoinositol depletion in rats with diabetes of longer duration. Extrapolation of results obtained in animal studies to humans is open to question in view of the limited number of treated diabetic patients (N = 21) with neuropathy and diabetic patients without neuropathy, (N = 4). In both these groups, nerve myoinositol levels were not decreased in response to increased sorbitol.

**EVENTS LEADING TO MYOINOSITOL DEPLETION**

INCREASED GLUCOSE AND SORBITOL

↓

LIMIT MYOINOSITOL ENTRY INTO CELL

LOW TISSUE MYOINOSITOL LEVEL

↓

CELL MEMBRANE PHOSPHOINOSITIDE SYNTHESIS REDUCED

↓

DIACYLGLYCEROL LEVEL LOWERED

↓

PROTEIN KINASE C ACTIVATION REDUCED

↓

SODIUM - POTASSIUM ATPase ACTIVITY LOWERED

↓

MYOINOSITOL TRANSPORT INTO CELL AFFECTED

↓

MYOINOSITOL DEPLETED.

*Figure 2. Events leading to myoinositol depletion.*
Methodology

**MEASUREMENT OF ALDOSE REDUCTASE ACTIVITY**

To a typical assay system consisting of 50 mM potassium phosphate (pH 6.0), 5 mM 2-mercaptoethanol as stabilizer, 0.4 M lithium sulfate as activator, and either 10 mM DL-glyceraldehyde or 5 mM D-glucose as substrate and 0.1 mM NADPH as coenzyme, a sample is added to bring total volume of reaction mixture to 1 ml. The decrease in absorbance at 340 nm as reduced nicotinamide adenine dinucleotide phosphate (NADPH) is monitored for four minutes at 37°C. One unit of aldose reductase activity is related to the oxidation of 1 micro mole of NADPH per minute at 37°C.

An assay mixture containing all the components except the substrate (glucose or glyceraldehyde) served as a blank. The enzyme has a requirement for sulfhydryl groups. Hence, a sulfhydryl activator such as 2-mercaptoethanol is included in the reaction mixture. The inclusion of lithium sulfate in the assay system results not only in a doubling of the apparent maximum velocity (Vmax) but also in an increase in the apparent Km values of DL-glyceraldehyde and NADPH by a factor of 4 to 5. Assay pH of 6.0 was chosen since it is closer to pH optimum of 5.5 for lens aldose reductase.

**MEASUREMENT OF POLYOLS**

Currently sensitive high performance liquid chromatographic (HPLC) procedures are available for the measurement of individual polyols such as sorbitol, galactitol, and myoinositol. In a typical HPLC procedure, steps involve solubilization of polyols in aqueous medium containing an internal standard such as glucose diethyl mercaptal, precipitation of protein with ethanol, and concentration of supernatant fluid prior to derivatization and analysis by HPLC. Phenylisocyanate that is used to derivatize polyols reacts with the hydroxyl group of polyols, thus forming an urethane bond. The HPLC column consists of octadecyl silica (C18 bonded silica) and elution of individual polyols from the column is effected with a solvent system consisting of acetonitrile:ethanol:water (reverse phase chromatography). The eluting polyols are monitored by U.V. detection at 340 nm. Sensitivity for individual polyols is 0.5 nMole.

Calibration curves for aqueous solutions of sorbitol, galactitol and myoinositol were linear in the range of 0.5 to 25 n.mole of sorbitol, 0.5 to 15 n.mole of galactitol, and 0.5 to 10 n.mole of myoinositol.

Precision of measurement of sorbitol, galactitol, and myoinositol as judged by multiple injections of derivitized tissue extracts (lens and sciatic nerve) of normal, diabetic and galactosemic rats were in the range of 1.0 to 2.1 coefficient of variation (C.V.). Number of replicate injections for each category was five.

Recovery studies conducted by addition of polyols to tissue extracts yielded recovery in excess of 91 percent.

**MEASUREMENT OF ALDOSE REDUCTASE INHIBITOR**

For the monitoring of therapeutic dose and assessment of drug toxicity, sensitive laboratory procedures are a prerequisite. For this purpose, HPLC procedures are ideal. Representative of such procedures is the method for the determination of Tolrestat an aldose reductase inhibitor that has shown promise in human clinical trials. In this procedure, serum is extracted with IN hydrochloric acid and isopropyl ether. The inhibitor in the organic phase is back extracted into glycine buffer at alkaline pH (pH 11), the aqueous phase neutralized with phosphoric acid and injected into a dual col-
umn HPLC apparatus containing octadecyl silica as stationary phase. The inhibitor is eluted from the column with acetonitrile in phosphate buffer at pH 6.0 and quantitated by measuring the absorbance in the U.V. at 226 nm.\textsuperscript{17}

The linearity of the recovery of tolrestat in the concentration range of 50 to 1000 ng per ml in human serum using replicate samples ($n = 3$ to 13) was 85.2 ± 1.1 percent. These recovery studies were performed on three different days, and day-to-day mean recoveries were not significantly different from those obtained for within-day recoveries. Since precise aliquots are used in the assay, the necessity for using an internal standard was obviated.\textsuperscript{17} Even so, to account for any minor variations in day to day recoveries, quantitation of samples in the analytical run was based on assaying spiked control sera which was carried through the procedure in the same manner as unknown samples. Correlation for peak height versus tolrestrat concentration in human serum in the range 50 to 1000 ng per ml was excellent ($r = 0.9980$). With human serum, the method had a detection limit of 25 ng per ml.

Drugs tested for potential interference in the method included indomethacin, hydrochlorothiazide, salicylic acid, acetaminophen, niacin, propoxyphene, diphenylhydantoin, phenobarbital, diazepam, tolbutamide, glyburide, phenylbutazone, and dicoumarol. Among these drugs, only phenylbutazone and dicoumarol produced strong interfering peaks.\textsuperscript{17}

Tolrestat was stable for at least three days when stored at room temperature, one week under refrigeration, and two weeks when frozen.

**Aldose Reductase Inhibitors**

These inhibitors can be categorized in four groups: carboxylic acids, hydantoins, flavonoids and other compounds. The majority of inhibitors that have been tested in human clinical trials belong to the category of carboxylic acids (epalrestat, statil, tolrestat and ponalrestat), with the exception of few inhibitors such as sorbinil which is an hydantoin. None of the flavonoids have reached the clinical trial stage.\textsuperscript{18} In figure 3 are presented the structures of selected inhibitors that have been used in human clinical trials.

**MECHANISM OF ALDOSE REDUCTASE INHIBITORS**

Apparently these inhibitors share similar structural characteristics, such as the presence of a carbonyl or thiocarbonyl group. These reactive groups allow the accepting of a pair of electrons from the enzyme, thus leading to its inhibition.\textsuperscript{22} In addition, the aromatic hydrophobic groups aid in the attachment of the inhibitor to the enzyme. Studies on the type of inhibition involved have come from typical experiments where each inhibitor is tested at several different concentrations against varying substrate (aldehyde) concentrations at saturating concentrations of coenzyme NADPH (0.1 mM). These experiments are repeated with varying concentrations of coenzyme NADPH at saturating concentration of aldehyde substrate (10 mM). Data were analyzed by conventional double-reciprocal Lineweaver-Burke plots ($1/V$ vs $1/S$) or other appropriate plots.

In a study that examined kinetics of inhibitors to human kidney aldose reductase, the inhibitor sorbinil exhibited non competitive inhibition (i.e., inhibitor binds at a site other than the active site of the enzyme, there by lowering maximum velocity $V_{\text{max}}$, but not affecting the binding of substrate to the enzyme or $K_{m}$) to both the aldehyde substrate and coenzyme NADPH.\textsuperscript{4} However, in that study two other inhibitors, Tolrestat and Statil, exhibited uncompetitive inhibition (inhibitor binds to the enzyme-substrate
complex thereby affecting both the Vmax and Km, characterized by parallel lines on the double reciprocal plot) towards the aldehyde substrate but exhibited like the inhibitor sorbinil noncompetitive inhibition to the coenzyme NADPH. The results of inhibitor kinetics of human kidney aldose reductase inhibitors are shown in Table II.

Figure 4 is a diagrammatic representation of inhibition plots diagnostic of noncompetitive and uncompetitive inhibition.

The amount of inhibitor required to achieve 50 percent inhibition of enzyme activity (the Ki or IC_{50}) varies from inhibitor to inhibitor. Thus, tolrestat has a Ki of 0.033 micro molar as compared to sorbinil with a Ki of 1.24 micro molar. As such, the doses of these inhibitors vary in general from 150 to 600 mg per day, with the exception of a drug in clinical evaluation in Japan (SNK-860) which has a dose of 7.5 to 30 mg per day.18

**Current Status of Aldose Reductase Inhibitor Therapy**

Of the inhibitors tested in human clinical trials, only two inhibitors have shown encouraging results. In a Japanese clinical trial using 214 patients with either nonproliferative or preproliferative diabetic retinopathy, the inhibitor epalrestat showed promise to the extent that treated patients had significantly less deterioration of the retinopathy.19 The other inhibitor which has shown promise is Tolrestat. One hundred twelve patients
TABLE II
Inhibitor Constants (KI) Derived for Human Kidney Aldose Reductase Inhibitors *

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Aldehyde (μM) Mean ± S.E.</th>
<th>NADPH (μM) Mean ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sorbinil</td>
<td>1.240 ± 0.160</td>
<td>0.510 ± 0.100</td>
</tr>
<tr>
<td>Tolrestat</td>
<td>0.033 ± 0.003</td>
<td>0.024 ± 0.003</td>
</tr>
<tr>
<td>Statil</td>
<td>0.001 ± 0.0003</td>
<td>0.007 ± 0.001</td>
</tr>
</tbody>
</table>


who received a 200 mg dose once daily demonstrated positive effects on peripheral nerve function. However, those treated with a 100 mg dose twice daily did not show improvement. Other studies, including effects of long-term use of drug on the eye and kidney, are in progress.

Some inhibitors such as sorbinil have dropped out of clinical trials because of hypersensitivity reactions evidenced by effects, such as skin rash. Thus, in one study only 72 percent of patients on treatment with sorbinil completed the study.¹ This study utilized 497 insulin dependent diabetes patients between the age of 18 to 56 who had either very mild or no diabetic retinopathy. Patients who received a dose of 250 mg per day of sorbinil for nearly three years failed to show an improvement in their condition of early diabetic retinopathy, leading to the speculation that the patient drop out rate could have had a bearing on the results of the study.¹,²,⁷ Other inhibitors, such as ponalrestat and statil, did not have a beneficial effect. Ponalrestat, administered to 62 diabetic patients at a high dose of 600 mg per day had no clinically significant effect on the progression of diabetic retinopathy.³

Another study in which ponalrestat was used on 259 diabetic patients with peripheral neuropathy for 18 months failed to show improvement.²⁹

Finally, the inhibitor, statil, tested on 47 neuropathic diabetic subjects at a high dose of 600 mg per day for 18 months failed to show a significant change in either nerve sorbitol concentrations or improvement in any morphometric parameter.¹⁵ The results of clinical trials of selected inhibitors of aldose reductase are summarized in table III.

Future Perspectives

Perhaps more needs to be known on the physiological role of the enzyme.
While the osmotic hypothesis explaining the changes ushering in cataract formation in lenses may be plausible, the implication of the role of myoinositol deficit needs to be established beyond doubt in view of some of the contradictory results that have been reported. It is to be noted that the polyols do not participate in the glycosylation of proteins, which leads to diabetic complications. In this context, aldose reductase inhibitors could conceivably be used to determine if diabetic complications are solely due to glycation.8 Aldose reductase inhibitors which have shown promise need to be thoroughly studied in carefully designed clinical trials on a large diabetic population. The clinical trials ought to last longer to assess effect of conditions such as retinopathy and neuropathy that take nearly 20 to 30 years to develop.38 One also needs to identify whether or not these inhibitors are effective only at early stages of diabetic complications, as some of the current studies would suggest. While there are well established laboratory methods for glycemic control, such as glycated hemoglobin10 and glycated albumin,24 precise methods should be available to assay for current and newly devised inhibitors to assess both the maintenance of therapeutic dose, compliance, and toxicity.

References


